Do leaf surface characteristics affect *Agrobacterium* infection in tea [*Camellia sinensis* (L.) O Kuntze]?

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The host range specificity of *Agrobacterium* with five tea cultivars and an unrelated species (*Artemisia parviflora*) having extreme surface characteristics was evaluated in the present study. The degree of *Agrobacterium* infection in the five cultivars of tea was affected by leaf wetness, micro-morphology and surface chemistry. Wettable leaf surfaces of TV1, Upasi-9 and *Kangra jat* showed higher rate (75%) of *Agrobacterium* infection compared to Upasi-10 and ST-449, whereas non-wettable leaves of *A. parviflora* showed minimum (25%) infection. This indicated that the leaves with glabrous surface having lower *q* (larger surface area covered by water droplet), higher phenol and wax content were more suitable for *Agrobacterium* infection. Caffeine fraction of tea promoted *Agrobacterium* infection even in leaves poor in wax (Upasi-10), whereas caffeine-free wax inhibited both *Agrobacterium* growth and infection. Thus, study suggests the importance of leaf surface features in influencing the *Agrobacterium* infection in tea leaf explants. Our study also provides a basis for the screening of a clone/cultivar of a particular species most suitable for *Agrobacterium* infection the first step in *Agrobacterium*-mediated genetic transformation.

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1. Introduction

Agrobacterium tumefaciens is a soil-borne bacterial pathogen of the family Rhizobiaceae which normally infects wounds of dicot plants at the root-shoot interface and causes severe damages due to the crown gall disease (Nesme *et al* 1987; Hawes *et al* 1989). The advent of recombinant DNA technology has enabled the use of *A. tumefaciens* for genetic transformation, despite the fact that there are many other efficient methods for direct gene transfer (Christou 1996). *Agrobacterium*-mediated transformation is the preferred method for its simplicity, cost effectivity, little re-arrangement of transgenes, ability to transfer relatively longer DNA segments (Hamilton *et al* 1996) and preferential integration of foreign genes into transcriptionally active regions (Ingelbrecht *et al* 1991) ensuring thereby proper expression of transgenes in transgenic plants (Hernandez *et al* 1999).

The limited host range specificity of *Agrobacterium* is a well documented fact (Nester *et al* 1984; Pueppke *et al* 1984; Hawes and Pueppke 1987; Hawes *et al* 1989). Even within a particular species, the range of host specificity varies widely amongst the different cultivars or genotypes (Hawes *et al* 1989), perhaps due to leaf micro-morphology, surface chemistry and degree of leaf wetness as in other host-pathogen interactions. These features are generally influenced by external environmental factors such as annual precipitation (rain, dew, fog/mist, relative humidity), temperature and light intensity (Evans *et al* 1992). The pronounced water repellency of leaf surfaces is an important factor because it prevents growth of epiphyllic microorganisms (Preece and Dickinson 1971). In contrast to this, excess leaf wetness promotes pathogen infection in many native and agricultural species (Reynolds *et al* 1989) and may be true in case of tea also. The outbreak and severity of foliar infection is governed by degree and

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duration of leaf wetness (Kim *et al* 2002). Continuous leaf wetness is also important for establishing significant levels of pathogen infection on the leaf surface (Chandler and Thomas 1991). Moreover, leaf wettability is governed by its micro-morphology and surface chemistry (Pandey and Nagar 2002, 2003). Infection of plants or explants by *Agrobacterium* is basically a host-pathogen reaction (DeCleene and DeLey 1976; DeCleene 1985) and leaves of tea cultivars with high levels of bacteriostatic polyphenols have been reported to be recalcitrant to *Agrobacterium* infection (Biao *et al* 1998). The leaf surface characteristics perhaps interfere with the process of cell-cell recognition and, hence, *Agrobacterium* infection. Therefore, it was necessary to identify and confirm whether micro-morphology, surface chemistry and wetness of tea leaves had any role to play in *Agrobacterium* infection. No such work has been done till date. Therefore, this work would be important for identification of suitable cultivars with competence for *Agrobacterium*-mediated transformations. The present study has been confined only to the first step of genetic transformation: i.e. attachment and growth of bacteria on the surface of the leaf explant.

2. Materials and methods

2.1 *Plant materials*

Leaves (1st leaf adjacent to the apical shoot bud, about 3·0 × 1·2 cm in size) from each of the five cultivars like *Kangra jat*, ST-449, Upasi-9, Upasi-10, Tocklai vegetative-1 of *Camellia sinensis* and non related species *Artemisia parviflora* with totally non-wettable leaf surface were employed as reference leaves for studying the relationship of leaf surface characteristics, micro-morphology and chemistry with *Agrobacterium* infection. These plant species were used for studying all the parameters. The leaf explants were obtained from tagged elite plants growing in the Institute's Experimental Farm, Palampur (1292 m above sea level, 32·6°N and 78·18°E).

Entire leaves without wounding were used in the present study in order to assess the infectivity of *A. tumefaciens*, since our previous studies showed that wounded or cut explants/leaves of tea were detrimental to *Agrobacterium* infection (Mondal *et al* 2001; Sandal *et al* 2002; Sandal 2003).

2.2 *Leaf micro-morphology and wettability*

Measurements were made on both adaxial and abaxial surfaces of leaf including stomatal trichome density (number mm^{-2}) and contact angles (leaf wettability, q) of the leaf surface. All the measurements were made on ten randomly selected healthy leaves from five different bushes/plants with three replications per leaf.

Stomatal and trichome density were calculated by counting their number with haemacytometer (1 × 1 mm grid) from surface impressions (clear enamel nail polish), and care was taken to avoid veins (Meidner and Mansfield 1968).

The degree of water repellency of the leaf surface was determined by measuring the contact angle (q) of a 2 mm^3 water droplet placed by micropipette on each leaf disc mounted on glass slides using double sided tape. Contact angle (q) is defined as the angle between the flat leaf surface and the line tangent to the water droplet through the point of contact and was measured according to Brewer *et al* (1991). For all leaves, q was measured relative to the epidermis for horizontally positioned leaf discs and criteria for judging surface wettability were based on those of Crisp (1963), where $q < 130$ as wettable and $q > 130$ as a non-wettable surface.

2.3 *Estimation of epicuticular wax and total phenols*

Epicuticular wax content was estimated according to Barnes *et al* (1996). Leaf discs of 10 mm diameter were selected for wax estimation and area of each disc was calculated using the formula πr^2. Both surfaces of leaf discs (0·79 cm^2 each) were washed separately with 10 cm^3 chloroform (HPLC grade) dispensed from a burette taking care not to contaminate the waxes extracted from one surface with another. Samples were filtered through pre-rinsed Whatman No. 1 filter paper and a 0·2 µm Sartorius filter into pre-weighed evaporating flask. The solvent was reduced under vacuum below 40°C before drying to constant weight at room temperature. The amount of wax obtained was expressed per unit leaf area. Decaffeinated wax was obtained by passing the wax through methylene chloride (dichloromethane) at 40°C to remove caffeine.

For total phenols, leaf tissue was homogenized in 0·3 N HCl in absolute methanol (2 : 1) and centrifuged at 10,000 rpm for 20 min at 5°C. The supernatant was evaporated to dryness and finally dissolved in 5 ml distilled water and total phenol was estimated using Folin's phenol reagent at 630 nm according to Swain and Hillis (1959). The amount of total phenol in the sample was calculated using standard curve prepared from tannic acid.

For caffeine, fresh tea leaves (100–500 g) were oven dried at 60°C to a constant weight and extracted overnight at room temperature in aqueous acetone. The extract was filtered and further extracted with 250 ml of n-hexane for the removal of lipids. The aqueous layer, thus obtained, was re-extracted with petroleum ether and ethyl acetate to remove catechins, and finally, with chloroform. The chloro-

form layer was concentrated to yield the caffeine fraction from tea leaves (patent filed in US, Sandal *et al* 2001).

2.4 *Effect of caffeine fraction, total phenols and wax on the growth of* Agrobacterium

Single cell colonies of *Agrobacterium* (strain EHA105 harbouring *hpt* and *gusint*) were grown in liquid yeast mannitol broth (YMB) medium (20 ml) containing 50 µg/ml hygromycin in the dark at 28°C for 16–18 h at 150–200 rpm. The effects of caffeine, phenol and wax (obtained as above) on the growth of *Agrobacterium* were also compared separately with untreated control. For this, bacterial cultures were grown in media supplemented with either caffeine (300 mg l^{-1}), phenol (50, 100 and 200 µg ml^{-1}), or wax with and without caffeine (50, 100 and 200 µg ml^{-1}). The concentration of caffeine was selected on the basis of our earlier work (Sandal *et al* 2001). Wax isolated from tea leaves was dissolved in chloroform and added to the liquid medium. The chloroform in the medium was allowed to evaporate in a laminar flow hood until no smell of chloroform was detected and the absorbance was recorded at 600 nm prior to inoculation. After inoculation, the differences between the absorbance values (recorded at 600 nm) of the (i) control containing only the bacteria, (ii) the control with the chemical compound but no bacteria and (iii) the bacterial culture in YMB supplemented with the chemical compound represented the growth of *Agrobacterium* and were plotted against time at regular intervals of 6 h for a total period of 24 h. Growth of *Agrobacterium* in response to these compounds was also estimated in terms of colony forming units on agar solidified YMB medium containing the above three groups of chemicals. The colonies formed on the agar solidified medium without the three groups of chemicals served as the control.

2.5 Agrobacterium *infection*

Intact leaves were surface sterilized following the method of Kumar (2003), and were inoculated on basal MS (Murashige and Skoog 1962) medium supplemented with 3% sucrose for 7 days to screen out aseptic explants.

Single cell colonies of *Agrobacterium* strain EHA105 harbouring the *hpt* and *gusint* genes were grown in liquid YMB medium (20 ml) containing 50 µg/ml hygromycin as mentioned above and cells corresponding to OD_{600nm} = 0·6 were pelleted by centrifugation at 6000 rpm for 10 min. The bacterial pellet was suspended in liquid YMB medium (10^9 cells/ml density) and leaf explants of tea cultivars and *A. parviflora* were submerged in it for 20 min and then blot dried on sterile filter paper and finally transferred to modified basal MS medium (agar solidified) for co-cultivation. For control, the leaves were submerged in bacteria-free YMB prior to co-cultivation. The leaves were then co-cultivated for 5 days and the percent infection or bacterial growth on the leaf surface was recorded as a percent of total leaves by using the formula: (infected leaves/total leaves) ∗ 100. In order to confirm whether the infection on the tea leaves was due to *Agrobacterium*, the overgrowing bacteria on the leaf explants were stained with Congo Red.

The effect of caffeine fraction (300 mg l^{-1}), and wax (200 mg l^{-1}) with and without caffeine (obtained from tea leaves) were also tested on the leaves of Upasi-10 (low wax content) to determine the rate of *Agrobacterium* infection. In a separate set of experiment, the epicuticular wax of the leaves of TV-1 (high wax content) were removed by peeling off the cuticle from the leaves with the help of a cello-tape and then used for *Agrobacterium* infection. Twenty replicates with ten leaves per replicate were used.

For confirmation of infection by *A. tumefaciens*, the leaves were tested for transient X-glu (5-bromo-4-chloro-3-indolyl-*b*-D-glucuronide) (GUS) expression following the method of Jefferson (1987). The leaves which tested positive for GUS were further subjected to PCR amplification using *npt*II and *gus* primers.

2.6 *Statistical analysis*

The effects of leaf wetness, micro-morphology, surface chemistry and plant species on *Agrobacterium* infection were analysed using analysis of variance (ANOVA) and the differences among means were tested by Duncan's multiple range test ($P < 0{\cdot}01$).

3. Results

3.1 *Leaf morphology*

Both *C. sinensis* and *Artemisia* showed significant differences in stomatal and trichome density, and *q* in relation to *Agrobacterium* infection. In all the five cultivars, stomata were present only on the abaxial (lower) surface whereas trichomes were present on both the surfaces of the leaf explant. Maximum number of stomata (284 mm^{-2}) and trichomes (8·4 mm^{-2}) were observed in ST-449 and Upasi-10 respectively, whereas minimum stomata (128 mm^{-2}) in Upasi-10 and trichome (3 mm^{-2}) in ST-449 were recorded. In general, higher *q* was observed on upper surface of leaves except in case of TV1 (figure 1C, D, E). The upper surfaces of leaves of ST-449 and Upasi-10 were comparatively less wettable ($q = 68$) followed by *Kangra jat* ($q = 67$) compared to Upasi-9 and TV1. On the other hand, the leaves of *A. parviflora* were observed to be highly non wettable ($q > 140$) and had higher number of trichomes and relatively higher number of stomata than all the cultivars of tea except ST-449 (figure 1C, D, E).

3.2 *Epicuticular wax and phenol*

The content of total phenol and wax of adaxial surface showed significant differences among all the plant species (figure 1A, B). The wax recovered from the abaxial surfaces of leaves of TV1 was significantly higher than all the other cultivars of tea. However, the lowest content of wax was recorded in *A. parviflora*. In general, the higher content of wax was recorded on the adaxial surface of leaves of all the plants tested as compared to the abaxial ones (figure 1B).

3.3 *Effect of caffeine fraction, total phenols and wax of tea leaves on the growth of* A. tumefaciens

Significant increase in bacterial growth both in growth curve and colony forming units (cfus) was observed with increasing concentrations of phenol, wax and caffeine fraction of tea leaves. However, it was maximum with increasing concentrations of phenol (figure 2A) compared to wax and caffeine which were at par after 12 h of growth (figure 2B, D). However, no bacterial growth was observed when caffeine free wax was added to the culture medium (figure 2C). Maximum increase of about 2·59 to 2·75-fold was observed with increasing concentrations of phenol at 12 h and 18 h respectively (table 1). However, in case of caffeine or wax, a similar trend in the growth of *Agrobacterium* was observed with increasing concentrations and time of incubation wherein the maximum increase noted was 1·5-fold at 18 h (table 1).

3.4 Agrobacterium *infection*

High rate of *Agrobacterium* infection (75%) was observed in the cultivars *Kangra jat*, Upasi-9, and TV1, whereas it was relatively low in ST-449 and Upasi-10 (50%) and least (25%) in the leaves of *A. parviflora* (figure 1).

When caffeine fraction (300 mg l^{-1}) was added to the co-cultivation medium of Upasi-10 leaves about 40% increase in *Agrobacterium* infection over control was observed (figure 3). Further, when leaves without cuticle were co-cultivated on medium supplemented with a combination of caffeine fraction and wax, a similar increase in infection was noted. In contrast to this, when leaves without cuticle were co-cultivated on medium supplemented with caffeine free wax, no infection was observed (figure 3).

When the cuticle of wax rich leaves of TV-1 was removed and subjected to *Agrobacterium* infection, a marked decrease (> 70%) in infection was observed (figure 3). Addition of caffeine fraction to such leaves, the infection increased by about 25% over control. However, when

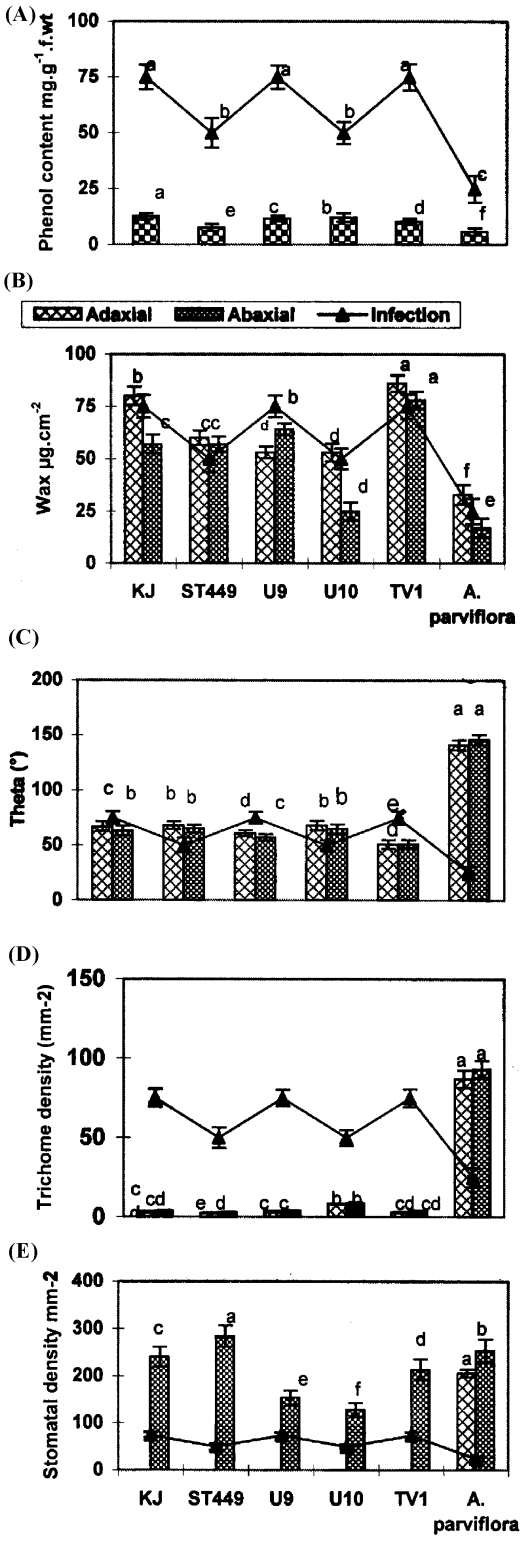

Figure 1. Leaf surface characteristics: (**A**) phenol; (**B**) wax; (**C**) contact angle (theta); (**D**) trichome density; and (**E**) stomatal density of tea (*C. sinensis*) and *A. parviflora*. The line above the histogram showed the percent *Agrobacterium* infection in these species. Different letters above the bars of the histogram indicate that the means are significantly different at $P < 0{\cdot}01$. Vertical bars ± SE.

leaves with cuticle were subjected to infection in the presence of caffeine fraction (300 mg l^{-1}), 33% increase was observed (figure 3).

The infected leaves when stained with Congo Red showed stained pinkish red colour confirming the presence of *A. tumefaciens*.

When GUS transient expression was tested in the leaves infected with *A. tumefaciens* (figure 4), high percent of leaves (9·9%) showed GUS expression in *Kangra jat* followed by that in Upasi-9 and TV1 i.e. 5·0 and 6·0% respectively (table 2). However, only 1–2% leaves showed GUS expression in Upasi-10 as compared to no expression in ST-449 (table 2). PCR amplification also confirmed the transformation of tea leaves by *A. tumefaciens* (data not shown).

Agrobacterium infection was found to be positively correlated with phenol (Spearman $r_s = 0{\cdot}78$; $P < 0{\cdot}01$) and wax (Spearman $r_s = 0{\cdot}86$; $P < 0{\cdot}01$). However, it showed significant negative correlation with trichome density (Spearman $r_s = -0{\cdot}82$; $P < 0{\cdot}01$) and q (Spearman $r_s = -0{\cdot}86$; $P < 0{\cdot}01$). No significant relationships of infection were found with stomatal density.

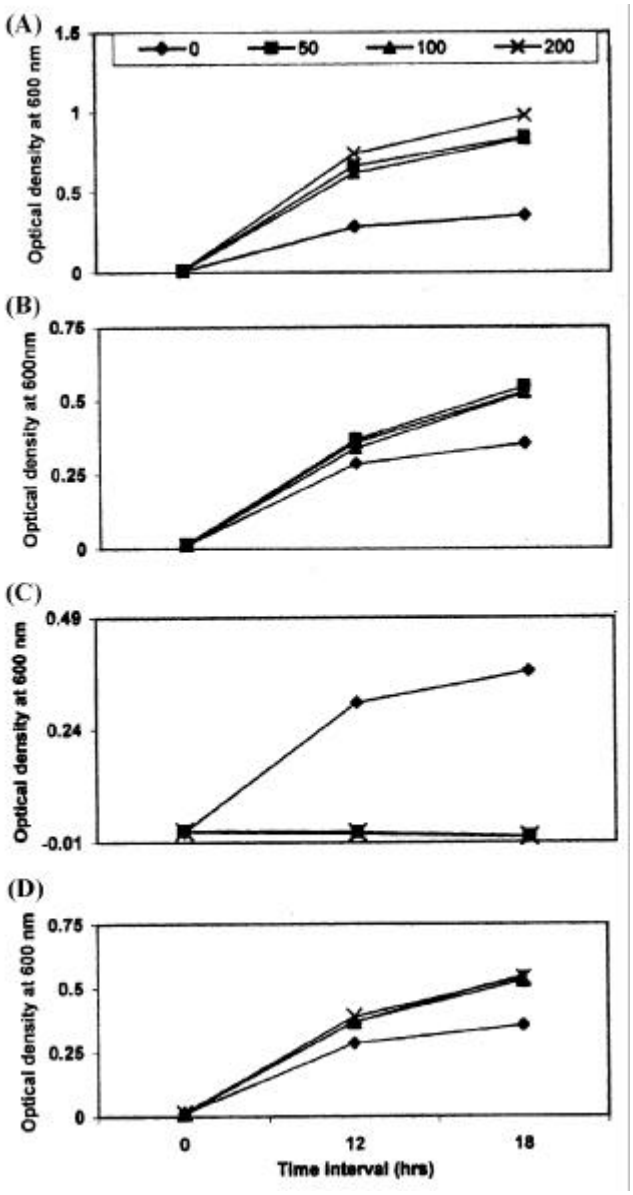

Figure 2. Effect of different concentrations of tea: (**A**) phenol; (**B**) wax; (**C**) caffeine-free wax; and (**D**) caffeine fraction on the growth of *A. tumefaciens* (◆ = 0 μg/ml; ■ = 50 μg/ml; ▲ = 100 μg/ml and × = 200 μg/ml).

4. Discussion

Besides the major factors responsible for cell-cell recognition, leaf surface chemistry and micromorphology were also hypothesized to be important for differential degree of *Agrobacterium* infection in different plant species. Generally, leaf surface characteristics govern the degree and duration of leaf wetness and are hence, considered to be responsible for the severity of pathogen attack (Kim *et al* 2002). This may also be true for *Agrobacterium*-host interactions especially because a considerable variation in *Agrobacterium* infection has been documented in a number of wild and economically important plant species (Hawes *et al* 1989). Variations in *Agrobacterium* infection (first step in *Agrobacterium*-mediated genetic transformation) in different explants of a particular species hold true for five different cultivars of tea (figure 1). This was further supported by GUS-transient expression analysis of leaves of five tea cultivars infected by *Agrobacterium*, which also exhibited variable degree of GUS expression (table 2).

Based on leaf surface chemistry and micro-morphology, two types of leaf surfaces i.e. wettable and non-wettable were identified. In the present study, the leaf surfaces with higher degree of wettability (lower q), phenol, wax and lower trichome density (< 5) showed high rates of *Agrobacterium* infection. Thus, 75% *Agrobacterium* infection was observed in the cultivars TV1, Upasi-9 and *Kangra jat*. On the other hand, the leaves of the cultivars Upasi-10 and St-449 showed relatively lower infection (50%) and were relatively less wettable. High phenol content in Upasi-10 with lower infection rate was probably due to relatively high trichome density and lower wax content but that in ST-449 was due to lower phenol as compared to other cultivars. Phenolic compounds are known to help in cell-cell recognition and *Agrobacterium* infection in plants (Ashby *et al* 1988; Zupan and Zambryski 1995) and this was also the case in tea where low concentrations of tea polyphenols (200 mg l^{-1}) promoted the growth of *Agrobacterium* (figure 2A). However, Biao *et al* (1998) reported the inability of *Agrobacterum* to

infect leaves of tea cultivars having high concentrations of polyphenol. Polyphenol concentration above 250 mg l^{-1} was reported to be bactericidal (Sandal 2003).

Trichomes are reported to increase the boundary layer thickness between the epidermal tissue and the environment by protecting plants against pathogen attack either by secreting chemicals (Karageorgou *et al* 2002) or by acting as a physical barrier against pathogen attack on vegetative tissues (Edwards 1992; Szymanski *et al* 2000). As per our observations, in general, each trichome covered roughly $0{\cdot}25 \times 10^{-3}$ mm^2 thus, in the leaves with higher density of trichomes (86–93/mm^2), the area covered was

Table 1. Effect of different chemicals extracted from tea leaves on growth and colony formation of *A. tumefaciens*.

Concentrations of chemicals extracted from tea leaves	Colony forming units after 48 h (cfus)	Fold increase in *Agrobacterium* growth over control after	
		12 h	18 h
Control	290 ± 15·32	1·00	1·00
Phenol			
50 µg/ml	800 ± 13·99	2·32	2·38
100 µg/ml	805 ± 16·87	2·20	2·35
200 µg/ml	1100 ± 20·87	2·59	2·75
Wax			
50 µg/ml	410 ± 12·65	1·28	1·54
100 µg/ml	413 ± 113·0	1·20	1·50
200 µg/ml	421 ± 11·43	1·25	1·49
Caffeine-free wax			
50 µg/ml	–	–	–
100 µg/ml	–	–	–
200 µg/ml	–	–	–
Caffeine fraction			
50 µg/ml	410 ± 12·76	1·29	1·53
100 µg/ml	413 ± 11·90	1·29	1·49
200 µg/ml	421 ± 14·02	1·35	1·52

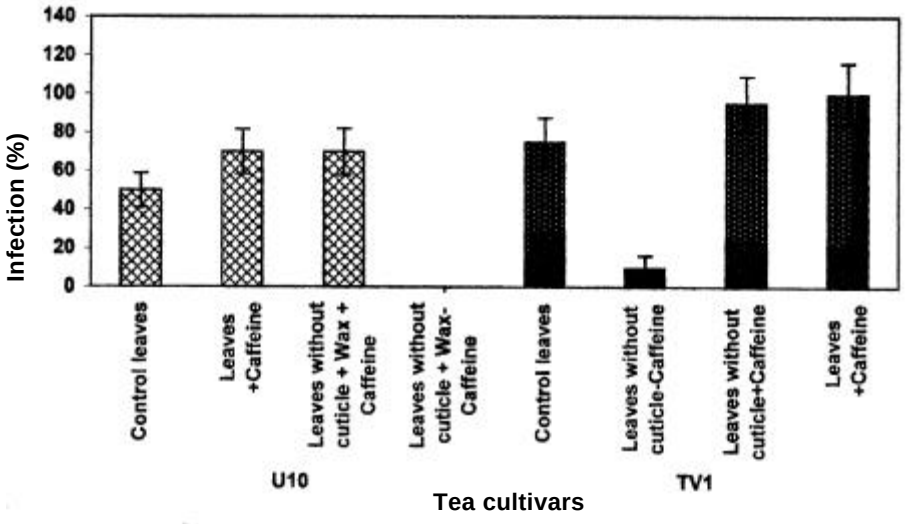

Figure 3. Effect of tea wax; caffeine-free wax and caffeine fraction on *Agrobacterium* infection of tea leaves with and without cuticle. Vertical bars ± SE.

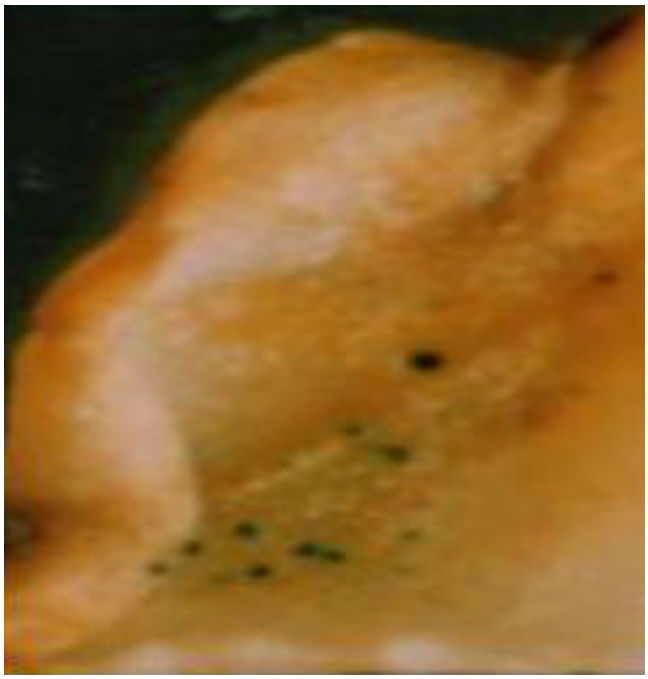

Figure 4. GUS-transient expression in tea leaf after *A. tumefaciens* infection.

Table 2. Percent leaves showing GUS transient expression in the five cultivars of tea after *A. tumefaciens* infection.

Cultivars	GUS expression (%)
Kangra jat (KJ)	9·9 ± 1·32
Stock-449 (ST-449)	Nil
Upasi-9 (U-9)	5·0 ± 0·87
Upasi-10 (U-10)	1–2 ± 0·12
Tocklai vegetative 1 (TV1)	6·0 ± 1·01

more ($2{\cdot}15 \times 10^{-2} - 2{\cdot}33 \times 10^{-2}$ mm^2) and the mean space among trichomes was very low (25–45 μm), as in *A. parviflora*. Approximately 28 times lesser area was covered in case of *Kangra jat* where the trichome density was low (3·2–3·5/mm^2) and the mean space among the trichomes was quite high (500–525 μm). Thus, more leaf surface area of vegetative tissues was available for the adherence of the *Agrobacterium* and larger space between the trichomes facilitated a larger number of *Agrobacteria* (4·4 × 1·6 μm, Hernandez 1999) in reaching the vegetative surface of leaf.

Wettability and other leaf surface characteristics play an important role in *Agrobacterium* infection. This has been further confirmed by infecting the non-wettable leaves of *Artemisia* where features like *q* and trichome density were high. In the present study, a droplet of 2 mm^3 wetted roughly 0·10 mm^2 of the non-wettable surface (*A. parviflora*, $q = 146$) and 0·33 mm^2 of the wettable surface (Upasi-9, $q = 57$). Upasi-9 also showed a high rate of *Agrobacterium* infection (75%). The relationship of higher '*q*' with lower *Agrobacterium* infection in the five cultivars of tea was probably due to the lesser surface area wetted by water droplets and this probably minimized the adherence of *Agrobacterium* to leaf surface because of its 'lesser stickiness'. For all the surfaces examined, '*q*' increased with increasing trichome density but it did not reflect any relation with stomatal density (figure 1). This is corroborated by the report of Juniper and Jeffree (1983) where no relation between stomatal density and leaf wettability was observed. All the cultivars of tea were hypostomatic. This is in agreement with the stomatal distribution pattern of terrestrial plants (Willmer 1983).

The aerial surface of all higher plants develop a layer of epicuticular wax on the outermost surface of cuticle, which along with other structures like trichomes form the first line of defense against external influences like air pollutants, high irradiance and attack by pest and pathogens (Percy *et al* 1994; Pandey and Nagar 2002). Thus epicuticular wax content was higher on the adaxial surface compared to abaxial ones in all cultivars of tea and *A. parviflora*. Although wax is generally negatively correlated with infection in most plants, a positive correlation between higher wax content and *Agrobacterium* infection was observed in tea cultivars. This can be explained by the reports of Mohammed *et al* (1986) who reported the presence of high caffeine in the wax of tea leaves and further Sandal *et al* (2001) found a positive effect of caffeine fraction on *Agrobacterium* infection. Promotion of *Agrobacterium* infection in the tea cultivars due to addition of caffeine fraction to the co-cultivation medium and no infection due to caffeine free wax in the present study was in accordance with the above report (figure 3).

The present study has several important implications in the field of crop improvement. 'Leaf surface-characteristics' can be used as an indicator for developing crops resistant to bacterial and fungal diseases in conventional breeding programs. Moreover, it can also be used as a valuable tool for screening potential explants/cultivars for *Agrobacterium*-mediated transformations. It is therefore suggested that 'leaf surface-characteristics' should be employed as an useful tool in crop improvement programs by way of conventional breeding and transgenic technology and also in understanding the dynamics of leaf wettability with pathogen infection or transgenic production through *Agrobacterium* infection. In this regard, the down or up regulation of the *CER* genes of *Arabidopsis thaliana* in the synthesis of epicuticular wax (Jenks *et al* 1995) or caffeine biosynthesis gene (Kato *et al* 2000) in tea may lead to novel genetic approaches to crop improvement.

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